REPORT DOCUMENTATION PAGE

DTIC FILE COPY

1a. REPORT SECURITY CLASSIFICATION		1b. RESTRICTIVE MARKINGS			
Unclassified					
2a. SECURITY CLASSIFICATION AUTHORITY	—	3. DISTRIBUTION / AVAILABILITY OF REPORT			
		Approved for public release; distribution is unlimited			
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE					
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		5. MONITORING ORGANIZATION REPORT NUMBER(S)			
NMRI 87-64					
6a. NAME OF PERFORMING ORGANIZATION	6b. OFFICE SYMBOL *(If applicable)*	7a. NAME OF MONITORING ORGANIZATION			
Naval Medical Research		Naval Medical Command			
6c. ADDRESS *(City, State, and ZIP Code)*		7b. ADDRESS *(City, State, and ZIP Code)*			
Bethesda, Maryland 20814-5055		Department of the Navy Washington, D.C. 20372-5120			
8a. NAME OF FUNDING / SPONSORING ORGANIZATION Naval Medical Research and Development Command	8b. OFFICE SYMBOL *(If applicable)*	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER			
8c. ADDRESS *(City, State, and ZIP Code)*		10. SOURCE OF FUNDING NUMBERS			
Bethesda, Maryland 20814-5055		PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
					N/A

11. TITLE *(Include Security Classification)*

Viable Legionella Pneumophila not Detectable by Culture on Agar Media

12. PERSONAL AUTHOR(S)

Hussong,D; Colwell,RR; O'Brien,M; Weiss,E; Pearson,AD; Weiner,RM; Burge,WD

13a. TYPE OF REPORT	13b. TIME COVERED		14. DATE OF REPORT *(Year, Month, Day)*	15. PAGE COUNT
journal article	FROM _____	TO _____	1987	4

16. SUPPLEMENTARY NOTATION

in: Bio-Technology v.5, n.9, 1987, pp.947-950

17. COSATI CODES			18. SUBJECT TERMS *(Continue on reverse if necessary and identify by block number)*	
GROUP	SUB-GROUP		LEGIONELLA	FLUORESCENT ANTIBODY
			LEGIONNAIRES'-DISEASE	VIABLE NON-CULTURABLE CELLS

AD-A200 898

19. (Continue on reverse if necessary and identify by block number)

DTIC SELECTED NOV 0 4 1988 D

DTIC COPY INSPECTED 6

20. DISTRIBUTION / AVAILABILITY OF ABSTRACT			21. ABSTRACT SECURITY CLASSIFICATION	
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS			Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL			22b. TELEPHONE *(Include Area Code)*	22c. OFFICE SYMBOL
Phyllis Blum, Information Services Division			202-295-2188	ISD/ADMIN/NMRI

DD FORM 1473, 84 MAR 83 APR edition may be used until exhausted. All other editions are obsolete.

VIABLE *LEGIONELLA PNEUMOPHILA* NOT DETECTABLE BY CULTURE ON AGAR MEDIA

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Detection and monitoring of genetically engineered microorganisms released to the environment, as well as pathogens, are primary factors in risk assessment. Culture methods have been proposed for both detection and monitoring. However, microorganisms in natural systems may not always be culturable. We surveyed environmental samples collected from sources implicated in an epidemic of Legionnaires' disease and, although no cultures were recovered from environmental samples, numerous cells were observed by fluorescent microscopy when anti-*L. pneumophila* group 1 antibody was used. Similar observations have often been made by others. To study this loss of culturability, *L. pneumophila* strains were maintained in a microcosm (vessels containing sterilized environmental water) and assayed at intervals for growth on appropriate media, and lethality for chick embryos. At 4°C, the decimal rate of decline of colony forming cells was approximately 29 days; at 37°C it was 13 days. When microcosm water samples were injected into embryonated eggs, far greater chick embryo mortality was observed than could be accounted for by the number of culturable cells in the injections. Thus, previously non-culturable *Legionella* had multiplied once again and become culturable. These results indicate that samples that do not contain culturable cells, may contain cells that are viable, as demonstrated by their pathogenicity for chick embryos. The fluorescent antibody assay may provide a valuable indication of the presence of such viable but non-culturable cells.

Genetically engineered microorganisms are being considered for a variety of environment applications. However, risks associated with the introduction of such microorganisms into the environment have been hypothesized, primarily extrapolated from cases where the release of non-genetically engineered organisms has resulted in adverse effects. Thus, detection and monitoring of genetically engineered microorganisms released to the environment becomes a prime consideration in risk assessment. The ability to culture bacteria has been the practical basis for determining their viability, and essential to assessing their presence since the time of Pasteur. Culturability, then, has been the method of choice for detection of microorganisms in the environment. In this sense, *Legionella pneumophila*, the agent of Legionnaires' pneumonia and related illnesses, poses a microbiological dilemma for environmental monitoring[1] and provides a useful paradigm for detection and monitoring of genetically engineered microorganisms in the environment. There is no indicator organism to suggest the presence of *L. pneumophila*, which necessitates the direct detection of *L. pneumophila* itself. Unfortunately, cultural recovery of *L. pneumophila* from environmental samples is hampered by the presence of faster growing bacteria, and guinea pig methods for the selective isolation of *L. pneumophila* require 10^6 cells[2-7]. *Legionella* have been shown to vary in antigenic composition, virulence and tolerance to chemical agents, such as chlorine. These differences have been correlated with the presence of plasmids[8,9], environmental source[10,11], and passage history[12]. Epidemiologic typing must, therefore, employ sera or monoclonal antibodies.

A direct fluorescent antibody method[13] was modified in our laboratory to provide quantitative estimates of *L. pneumophila* (serogroup 1) in environmental samples. Some of the samples were collected from the Stafford District General Hospital, shortly after an outbreak of Legionnaires' pneumonia. Although no cultures were recovered from the environmental water samples after the Stafford epidemic, numerous *L. pneumophila* of the epidemic type were identified. We hypothesized that these bacteria may have been viable but non-culturable, as hypothesized for *Vibrio cholerae* and related organisms by Colwell et al.[14] Experiments were conducted in laboratory models (microcosms) to test this hypothesis for *L. pneumophila*.

RESULTS

Figure 1 illustrates the growth and survival of Philadelphia 2 in a sterilized tap water microcosm held at 37°C, as measured by colony count (CFU), acridine orange direct count (AODC)[15] and direct fluorescent antibody (DFA) count. A brief period of growth, evident by all measurements, was followed by declining counts. The colony counts began to decline by the tenth day and AODC counts declined after the twentieth day. DFA counts remained near their peak level following the growth period: 3×10^4/ml (day 5) to 1×10^4/ml (day 37). Decimal reduction time (D) for the CFUs was estimated to be approximately 15 days.

Figure 2 illustrates the CFUs of strain 62210 (a clinical isolate from the Stafford epidemic) held at 4°C and 37°C. The regression lines drawn for both temperatures indi-

cate D values of 13 days at 37°C, and 29 days at 4°C. The last culturable colonies from the microcosm maintained at 4°C were obtained from the 74th day sample (2.7 CFU/ml). The last culturable colonies from the microcosm maintained at 37°C were obtained from the 40th day sample (1.2/ml). DFA counting of a 37°C sample indicated 7000/ml on day 32, at a time when the CFU count was 25/ml.

The decline in culturable populations of *Legionella* in microcosms appeared to be temperature dependent. However, DFA counts showed much less or no decline (Figs. 1 and 2). Additionally, the AODC of *L. pneumophila* strain Philadelphia 2 in tap water (Fig. 1) indicated that many cells retained properties of viability long after they were no longer detected by agar culture.

To further investigate the viability of nonculturable cells, guinea pigs were inoculated intraperitoneally with freshly agar-grown *L. pneumophila* strain 62210. The LD_{50}, calculated by the Reed-Muench method, was 1.6×10^6 CFU, indicating that the method was not sufficiently sensitive for the detection of subtle changes in viability of cells in the microcosms. For this reason, the more sensitive embryonated egg yolk sac inoculation was used to determine the LD_{50} ($YSLD_{50}$) before and after microcosm treatment. The $YSLD_{50}$ of strain 62210 and Philadelphia 1 washed from fresh agar growth, was, in each case 10 CFU, a much greater sensitivity than the guinea pig procedure.

Figure 3 illustrates the $YSLD_{50}$ of strain 62210 before microcosm treatment, following brief microcosm treatment at 4°C and following extended incubation at 4 and 37°C. Brief incubation (2 weeks) in a 4°C microcosm resulted in no change in the $YSLD_{50}$ to CFU ratio, i.e. $YSLD_{50} = 10$ CFU (Log=1.0). Following microcosm treatment, the $YSLD_{50}$ for 4°C microcosm cells was 0.5 CFU (Log=−0.3), and the $YSLD_{50}$ for 37°C microcosm cells was 0.25 CFU (Log=−0.6). Thus, the $YSLD_{50}$ values were reduced 20- to 40-fold from the values for fresh cultures of strains 62210 (Fig. 3) or Philadelphia 1 (data not shown) washed from agar, or for *Legionella* suspended for only 2 weeks in 4°C tap water.

Yolk sac tissue from lethally infected chick embryos was examined microscopically and revealed many bacteria that stained intensely with acridine orange, even from eggs inoculated with less than 1 CFU (Fig. 4). In addition, agar culture of yolk sac tissue from these embryos permitted dense growth of *L. pneumophila*. These results showed lethal infections were regularly occurring from inocula containing less than one culturable bacterium. Thus, previously non-culturable bacteria had become culturable following infection of chick embryos.

Culture 62210 was examined by indirect fluorescent antibody for its monoclonal antibody binding pattern[16,17] (Table 1). Patterns for additional isolates from other Stafford sources were also determined, as were patterns for bacteria in environmental water samples (which were suspected as the source of the epidemic, but did not yield culturable *Legionella*). Antigens detected on bacteria in the environmental samples revealed a monoclonal antibody type similar to strain 62210, but additional antigens were detected as well (Table 1). From a 37°C microcosm incubated for 40 days, cells of strain 62210 did not change in antigen presentation from the original culture of 62210 (Table 1). Thus, incubation in tap water did not induce antigenic changes, and this treatment does not explain the variety of antigens on the cells collected in the Stafford condensate water.

DISCUSSION

The loss of the culturable population of *L. pneumophila* in the microcosms appeared to be temperature depen-
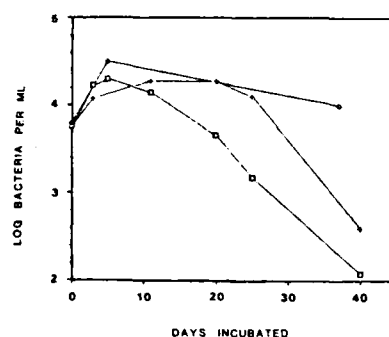

FIGURE 1 Growth and survival of *L. pneumophila* strain Philadelphia 2 at 37°C in sterilized tap water. Colony forming units on pbCYEa agar (squares); Acridine orange direct count (crosses); Direct fluorescent count (diamonds).
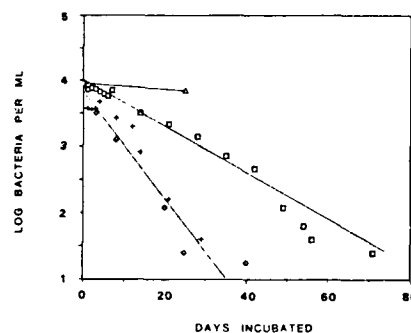

FIGURE 2 *L. pneumophila* strain Stafford 62210 in tap water microcosms held at 4°C and at 37°C (duplicate). Colony count of cells from 4°C (squares), and 37°C (crosses and diamonds). Direct immunofluorescent count of *L. pneumophila* in the 37°C microcosm (triangle).
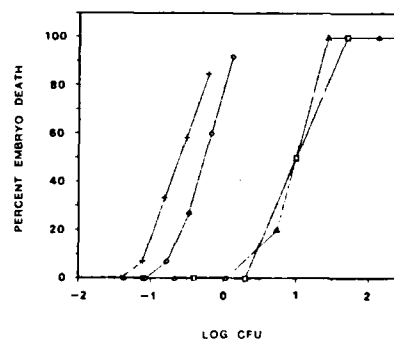

FIGURE 3 Yolk sac lethal dose titration for *Legionella* strain Stafford 62210. The percentages of embryo deaths were calculated by Reed-Muench method. Log of the CFU injected into yolk sacs is shown on the x-axis. Bacteria washed from fresh agar culture (triangles), maintained for two weeks in tap water at 4°C (squares); maintained 40 days in 37°C tap water (crosses); maintained 72 days in 4°C tap water (diamonds).

dent. DFA counts of the Philadelphia 2 and one of the 37°C strain 62210 microcosms indicated no decline beyond the error inherent in these counts. We conclude the DFA remained fairly stable while the CFU declined, and the AODC of Philadelphia 2 in tap water suggested that these cells remained viable.

We had tried to use the direct viable count (DVC) with yeast extract enrichment and nalidixic acid treatment to prevent cell division[18]. Unfortunately, the *Legionella*

strains tested were found to lyse under these test conditions. Without some form of DVC, we were unable to further refine our estimate of the proportion of viable but non-culturable cells in the microcosm samples.

No change in antigen presentation was detected by IFA with monoclonal antibodies, following microcosm treatment. This was not surprising since strain 62210 did not exhibit growth in the microcosm (as did Philadelphia 2), and would not be expected to synthesize new surface antigens.

Results from inoculations of embryonated eggs with the microcosm samples showed that viable L. pneumophila were culturally recoverable from yolk sac tissue of lethally infected embryos inoculated with less than one culturable bacterium, which is one tenth the infectious dose of culturable bacteria derived from agar. For the sake of estimation, it can be calculated that 1 to 0.5% of the DFA positive bacteria in the 37°C microcosm were viable, if the $YSLD_{50}$ (per cell, not CFU) for strain 62210 remained the same after microcosm treatment as it was in the control tests, 10 cells. It seems probable, however, that the virulence of these non-culturable bacteria was reduced[8,10,11], and the number of bacteria required to kill 50% of the chick embryos was somewhat greater.

McDade and Shepard[7] described virulence conversion of L. pneumophila cultured under different conditions, and showed that the most selective method of recovery was guinea pig inoculation. Their data also showed poor recovery of Legionella on enriched Mueller-Hinton (MH-1H) agar and slightly better recovery on Feeley-Gorman (FG) agar. Since these methods were not sensitive, even for infected tissue, these agar media were not employed for environmental recovery.

When Feeley et al.[10] employed charcoal yeast extract (CYE) agar, excellent sensitivity was observed for all but one test situation where agars were inoculated with infected tissues. In that case, although recovery was accomplished, numerical recovery on agar was only 1% the count predicted by DFA. In that study, they proposed a different physiologic state to explain the discrepancy in counts, as well as the possibility that the DFA counts arose from cells killed during the tissue grinding and harvesting procedure.

Our data show similar recoveries from a model environ-ment as was previously observed in tissues by Feeley et al.[18], but we have been able to show that the cells not recovered actually remained viable. Thus we demonstrate the modified physiology of non-culturable L. pneumophila in tap water is a viable but non-culturable state. Bacteria in this state, may account for a large proportion of environmental bacteria, and could well account for the often observed failure to culture Legionella from suspect samples[20]. Obviously other factors, such as large numbers of background flora, also take their toll in recovery.

Although bacterial "die-off" in the environment has been reported often when cultural assays are used, recent reports have suggested that direct microscopic counting may offer more valuable estimates when the bacteria to be counted may persist in a dormant phase awaiting suitable conditions for outgrowth[14]. The implications of the "viable but non-culturable state" in V. cholerae for release of genetically engineered organisms to the environment have been presented by Colwell et al.[14] The embryonated egg studies reported here show that L. pneumophila is yet another bacterial species (Vibrio cholerae, Escherichia coli, Salmonella and Shigella sp. and Campylobacter jejuni)[21,22] which may persist as viable, but non-culturable by direct
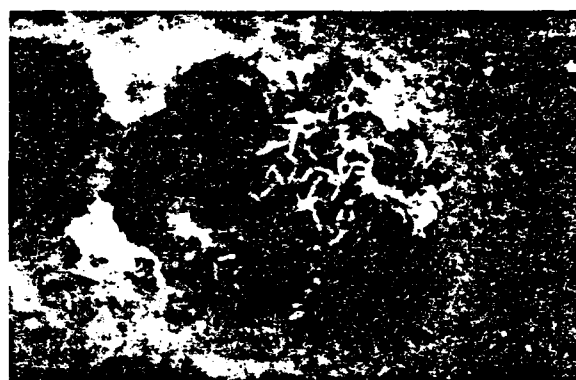

FIGURE 4 Metabolically active bacteria are observed by fluorescent microscopy of acridine orange stained yolk sac tissue harvested from a hen egg embryo which had been inoculated with 0.6 CFU of L. pneumophila strain 62210 from a 37°C microcosm incubated 40 days.

TABLE 1 Immunofluorescent assay with monoclonal antibodies to L. pneumophila.

Sample[2]	Monoclonal antibodies[1]											
	W29	P4C3	P9C3	P13-15	W36	W32	JR5	4032	W39	1A	1B	2A
Cultures												
Phila. 1	−	−	+	−	+/−	−	++	−	−	++	+	−
62210	−	−	+	+/−	++	−	−	−	−	+	−	−
62211	−	−	+	+/−	+	−	−	−	−	++	−	−
62212	−	−	++	+/−	+	−	−	−	−	++	−	−
62213	−	−	+	+	+	−	−	−	−	++	−	−
Togus	−	−	−	−	−	−	−	−	−	−	−	+
Stafford drip tray condensate												
43036	+	+	++	+/−	+	−	++	+	−	+	++	·
43037	+	+	++	+/−	+	−	++	+	−	+	++	−
43038	+	+	++	+/−	+	−	! +	+	−	+	++	−
43039	+	+	+	+/−	+/−	−	++	+	−	+	++	−
43040	+	++	+	+/−	+	−	+	+	−	+	++	−
43041	+	+	+	+/−	+	−	++	+	−	+	++	−
Tap water microcosm												
cells	−	−	++	+/−	++	−	−	−	−	+	−	−

[1]Monoclonal antibodies against serogroup 1 strains of L. pneumophila were obtained from Watkins and Tobin[15], except antibodies 1A and 1B (anti-serogroup 1, Philadelphia 2 strain) and 2A (anti-serogroup 2, Togus strain) which were prepared in our laboratories.
[2]L. pneumophila strains Philadelphia 1 and Togus served as controls. Environmental samples of condensate waste drainage from drip-collection trays were a potential source of the epidemic agent. Bacteria in the tap water microcosm at 37°C were concentrated by centrifugation and IFA stained.

culture methods. With newly described DFA reagents now available for *L. pneumophila*, false-positive reports due to cross-reactive bacteria will be greatly reduced[23]. In the future, greater confidence should be placed in DFA results, even in the absence of cultural confirmation. Reliable and accurate detection methods, other than cultural, are needed for genetically engineered organisms released to the environment. Until a suitable assay using gene probes and related molecular genetic methods for detection and monitoring are available, the immune fluorescent-epifluorescent detection method appears to be the most effective.

EXPERIMENTAL PROTOCOL

Legionella culture. *L. pneumophila* Philadelphia 1 was received frozen in a suspension of guinea pig spleen tissue prepared at the Centers for Disease control. The culture was grown on phosphate buffered charcoal yeast extract agar with alpha-ketoglutarate[21] (pbCYEa) for a single passage, suspended in 20 mM potassium phosphate[25], pH 6.9 buffer with 20% glycerol, and frozen in liquid nitrogen vapor. Philadelphia 2 is described elsewhere[21], and was received frozen in phosphate buffer. Isolates from Stafford (62210, 62211, 62212 and 62213) were received lyophilized, grown through two passages on pbCYEa and frozen in liquid nitrogen. CFU counts were done on pbCYEa by spreading 0.1 or 0.2 ml of diluted sample over the surface of pbCYEa agar plates. Plate count agar (Difco Laboratories) enriched with yeast extract to 1% final content plus ferric pyrophosphate enrichment (PCAYE) was also inoculated to detect contamination. Cultures were incubated in a 37°C humidified incubator with 2 to 3% CO_2 for 24 to 48 hours for seed cultures, and up to 7 days for CFU counts. *L. pneumophila* in yolk sac tissue were cultured by smearing washed tissue over pbCYEa. PCAYE and thioglycollate broth were also inoculated for controls.

Microscopic counts. DFA reagents were provided by the Center for Disease Control and samples were stained according to their instructions[26]. Samples for AODC were filtered onto Nuclepore 0.22 μm membranes and stained as described elsewhere[15]. The Kogure modification[15] (DVC) of the AODC was attempted on 8 control strains *Legionella* in the laboratory. Epifluorescent microscopy was performed with a Nikon 100 watt mercury illumination system, filtered by blue excitation and "K" prefilter for fluorescein.

Laboratory microcosms. Tap water was autoclaved in acid-washed flasks as previously described[11,21]. *L. pneumophila* from frozen stocks, were cultured on agar until the earliest evidence of growth was easily visible. Cells were washed from the agar in 20 mM potassium phosphate, pH 6.9 and resuspended to an optical density of 0.1 at 420 nm. The suspension was diluted 1/10 and 1 volume was added per 1000 volumes of microcosm. Microcosms were incubated with a slow stirring device to maintain uniformity.

Lethal dose titrations. Six day old embryonated eggs were inoculated with 0.5 ml of microcosm samples or their dilutions in phosphate buffer. Eggs were candled at three days for non-specific death and daily thereafter for 14 days. Dead embryos were refrigerated until the 14th day when the yolk sac tissues were harvested[3]. Yolk sacs were examined by AODC and cultured to confirm death by *Legionella* infection. Only confirmed infections were counted for calculation of $YSLD_{50}$ by the Reed-Muench method. Guinea pigs were injected intraperitoneally with washed suspensions of strain 62210 at decimal dilutions. Animals were checked daily for evidence of infection or death[3]. Autopsies were performed to confirm by culture that deaths were due to *L. pneumophila*.

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